

REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

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1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED	
	13 July 2009	Final Report	
4. TITLE AND SUBTITLE		5. FUNDING NUMBERS	
Host Immune Response to Bacterial Cyclic Diguanlylic Acid (c-di-GMP)		W911NF-07-1-0009	
6. AUTHOR(S)		8. PERFORMING ORGANIZATION REPORT NUMBER	
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
University of MD, Baltimore, 685 W. Baltimore Street, Baltimore, MD 21201; Intronetics Research Institute, 415 Oakington Road, Havre de Grace, MD 21078			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)		12 a. DISTRIBUTION / AVAILABILITY STATEMENT	
U. S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211		12 b. DISTRIBUTION CODE	
11. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.			
12 a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.		13. ABSTRACT (Maximum 200 words) This work tested the hypothesis that c-di-GMP is a novel immunostimulatory molecule that modulates the immune response. Intramammary treatment of mice with c-di-GMP before S. aureus challenge gave a protective effect and a 10,000-fold reduction in CFUs in tissues. Intramuscular vaccination of mice with c-di-GMP coinjected with S. aureus clumping factor A (ClfA) Ag produced serum with significantly higher anti-ClfA IgG Ab titer compared with ClfA alone. Intraperitoneal injection of mice with c-di-GMP activated monocyte and granulocyte recruitment. Human immature dendritic cells (DCs) cultured in the presence of c-di-GMP showed increased expression of costimulatory molecules CD80/CD86 and maturation marker CD83, increased MHC class II and cytokines and chemokines such as IL-12, IFN- γ , IL-8, MCP-1, IFN- γ -inducible protein 10, and RANTES, and altered expression of chemokine receptors including CCR1, CCR7, and CXCR4. c-di-GMP-matured DCs demonstrated enhanced T cell stimulatory activity. We found that c-di-GMP activates ERK phosphorylation in human M-CSF-induced monocyte-derived macrophages. c-di-GMP is stable in human serum. We have also found that c-di-GMP acts as a potent adjuvant in vaccine studies in vivo to inhibit infection by MRSA, Streptococcus pneumoniae, and Klebsiella pneumoniae. We propose c-di-GMP can be used clinically as an immunomodulator, immune enhancer, or vaccine adjuvant.	
14. SUBJECT TERMS c-di-GMP, vaccine, immunomodulator, adjuvant, infection		15. NUMBER OF PAGES	
		16. PRICE CODE	
17. SECURITY CLASSIFICATION OR REPORT <i>UU</i>	18. SECURITY CLASSIFICATION ON THIS PAGE <i>UU</i>	19. SECURITY CLASSIFICATION OF ABSTRACT <i>UU</i>	20. LIMITATION OF ABSTRACT <i>UU</i>

NSN 7540-01-280-5500

Standard Form 298 (Rev.2-89)
Prescribed by ANSI Std. Z39-18
298-102

Enclosure 1

Final report
AMSRD-ARL-RO-OI Proposal Number:50526-LS
James P. Nataro, MD PhD
David R. Karaolis, PhD
July 2009

Introduction

C-di-GMP is a naturally occurring small dinucleotide originally identified as a bacterial signaling molecule (2,4,6). Studies *in vitro* have shown that c-di-GMP has antimicrobial properties and the capacity to activate cells of the immune system. Specifically, c-di-GMP has been shown to activate macrophages and dendritic cells (1,5,7). Preliminary data from Dr. Davis Karaolis (University of Maryland) indicate that c-di-GMP has adjuvant/immunomodulatory properties when co-administered with microbial antigens to adult mice. Due to its capacity to induce maturation of dendritic cells and secretion of Th1-type cytokines - key elements to trigger immune responses in the neonatal setting-, we believe this molecule can serve as an effective adjuvant and vaccine delivery system for young hosts.

We have previously demonstrated that exogenous c-di-GMP significantly reduces *in vitro* cell-cell interactions and biofilm formation of *Staphylococcus aureus*, including human methicillin-resistant *S. aureus* strains and animal clinical isolates (6,19). We have also previously provided *in vivo* proof-of-concept that treatment with c-di-GMP (200nmol) significantly attenuates *S. aureus* infection and reduces bacterial cell number (10,000-fold; $p < 0.001$) in a mouse model of infection. The results of this study imply that c-di-GMP may have a biological effect on the *in vivo* environment and the host immune response. The purpose of this research program is to further test the hypothesis that c-di-GMP is not only an important microbial signaling molecule, but is also a novel immunostimulatory agent that can modulate the host immune response.

Overall Progress: We have made very good progress in our studies. Specifically for this proposal, we proposed studies that further explore the immunodulatory properties of c-di-GMP. The ***central hypothesis*** of this proposal is that **c-di-GMP is a novel immunostimulatory agent** and we sought to better understand the molecular mechanism of action of c-di-GMP on the host immune response and its potential clinical application.

Aim 1:

Specific Aim 1a: We proposed to further explore our preliminary data suggesting that c-di-GMP is an immunostimulatory molecule. In these studies, we will use DCs to study further the effect of c-di-GMP on the expression of surface markers and cytokines of cells treated with c-di-GMP to determine an overall profile.

Specific Aim 1 b. We proposed to study if the mechanism of action of c-di-GMP on the immune response involves Toll-like receptors since TLRs are known to be important receptors for immunostimulatory ligands, including microbial products.

Specific Aim 1c: We proposed to study whether c-di-GMP interacts with Nods since Nods are cytosolic proteins involved in innate immune defense, through pathways that are likely to be independent of TLR signaling.

Specific Aim 1d: Since it has been reported that activation of p38 MAPK is important for the differentiation/maturation of DCs and NF-kb has been linked to the production of

proinflammatory cytokines by DCs, we proposed to determine whether the mechanism of action of c-di-GMP on the host immune response might involve p38, ERK, JNK and NF- κ B signaling pathways.

Accomplishments for Aim 1: We have found that c-di-GMP is indeed an immunomodulator and immunostimulatory molecule. Intramammary treatment of mice with c-di-GMP 12 and 6 h before *S. aureus* challenge gave a protective effect and a 10,000-fold reduction in CFUs in tissues ($p < 0.001$). Intramuscular vaccination of mice with c-di-GMP coinjected with *S. aureus* clumping factor A (ClfA) Ag produced serum with significantly higher anti-ClfA IgG Ab titer ($p < 0.001$) compared with ClfA alone. Intraperitoneal injection of mice with c-di-GMP activated monocyte and granulocyte recruitment. Human immature dendritic cells (DCs) cultured in the presence of c-di-GMP showed increased expression of costimulatory molecules CD80/CD86 and maturation marker CD83, increased MHC class II and cytokines and chemokines such as IL-12, IFN- γ , IL-8, MCP-1, IFN- β -inducible protein 10, and RANTES, and altered expression of chemokine receptors including CCR1, CCR7, and CXCR4. c-di-GMP-matured DCs demonstrated enhanced T cell stimulatory activity. c-di-GMP did not activate NF- κ B, but activated p38 MAPK (as indicated by the increase in the phospho-p38 in DCs at both 5- and 20-min treatment). We found that c-di-GMP (10 and 100 nM) activates ERK phosphorylation in human M-CSF-induced monocyte-derived macrophages (Fig. 9B). The magnitude of c-di-GMP activation of ERK phosphorylation was weaker than that by LPS under the conditions tested. c-di-GMP is stable in human serum. We found that neither Nod1 or Nod2 responded to c-di-GMP. These results suggest that c-di-GMP immunostimulation does not involve any known TLR or Nod. We propose that cyclic dinucleotides like c-di-GMP can be used clinically in humans and animals as an immunomodulator, immune enhancer, immunotherapeutic, immunoprophylactic, or vaccine adjuvant.

These findings have been published: *Journal of Immunology*, 2007, 178: 2171–2181. See Appendix 1.

Specific Aim 2: This aim will address whether c-di-GMP can be used clinically to inhibit infection in several mouse models. These studies are underway.

Accomplishments for Specific Aim 2a: Sepsis model studies are scheduled to be performed in September, 2007

Accomplishments for Specific Aim 2b: Wound abscess model is scheduled to be performed in September, 2007.

Accomplishments for Specific Aim 2c: Rabbit indwelling medical device model is scheduled to be performed in September, 2007.

Progress in additional studies:

c-di-GMP Stimulates Protective Innate Immunity in Bacterial Pneumonia

We have previously shown that c-di-GMP is a unique small molecule immunomodulator and immunostimulatory agent triggering protective host innate immune responses. Using a murine model of bacterial pneumonia, we show that local intranasal (i.n.) or systemic subcutaneous (s.q.) administration of c-di-GMP prior to intratracheal (i.t.) challenge with *Klebsiella*

pneumoniae stimulates protective immunity against infection. Specifically, i.n. or s.q. administration of c-di-GMP 48- and 24 h prior to i.t. *K. pneumoniae* challenge resulted in significantly increased survival. Pretreatment with c-di-GMP resulted in a 5-fold reduction in bacterial CFU in lung ($p<0.05$) and an impressive >1000 -fold decrease in CFU in the blood ($p<0.01$). c-di-GMP administration stimulated a robust innate response to bacterial challenge, characterized by enhanced accumulation of neutrophils and $\alpha\beta$ T cells, as well as activated NK and $\alpha\beta$ T lymphocytes, which was associated with earlier and more vigorous expression of chemokines and type-1 cytokines. Moreover, lung macrophages recovered from *Klebsiella*-infected mice pretreated with c-di-GMP expressed greater quantities of iNOS (inducible nitric oxide synthase) and nitric oxide ex-vivo than did macrophages isolated from infected mice pretreated with control c-GMP. These findings demonstrate that c-di-GMP delivered in either a compartmentalized or systemic fashion stimulates protective innate immunity in the lung and protects mice against bacterial invasion. We propose that the cyclic dinucleotide c-di-GMP can be used clinically as an effective immunomodulator, immune enhancer and vaccine adjuvant to protect against respiratory infection and pneumonia in humans and animals.

These findings have been accepted for publication: *Infection and Immunity*, 2007, in press. Appendix 2.

c-di-GMP is an Effective Immunostimulatory Molecule Against Pneumococcal Infection
Streptococcus pneumoniae is the leading cause of bacterial pneumonia, meningitis, and otitis media in the United States. In spite of the availability of antimicrobials, the capsular polysaccharide (PS) vaccine, and the 7-valent protein-PS conjugate vaccine, pneumococcal disease continues to be responsible for high morbidity and mortality worldwide, especially in groups at high risk. To date, the most promising vaccine candidates are the pneumolysin toxoid (PdB), pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC, also referred to as choline binding protein A) and the 37-kDa metal-binding lipoprotein PsaA. Current global efforts are focused on alternative prophylactic strategies against pneumococcal disease. c-di-GMP (cyclic diguanylate) is a novel bacterial intracellular signaling molecule capable of stimulating protective host innate immunity against a variety of bacterial infections. The effects of intranasal administration of c-di-GMP before intranasal challenge, or intraperitoneal coadministration of c-di-GMP with the pneumolysin toxoid (PdB) or PspA before intraperitoneal challenge with *pneumococci* were investigated in mice. We show that intranasal pretreatment of mice with c-di-GMP resulted in significant decrease in bacterial load in the lungs and blood after serotype 2 challenge, significant decrease in lung titers after serotype 4 challenge, and significant reduction in bacterial counts after serotype 3 challenge. Potential cellular mediators of these protective responses have been identified in lungs and draining lymph nodes. Furthermore, intraperitoneal coadministration of c-di-GMP with PdB or PspA before challenge resulted in significantly higher antigen-specific antibody responses, protection and increased survival of mice, compared to that obtained with alum adjuvant. These findings demonstrate that local or systemic c-di-GMP administration stimulates protective innate immunity and protects mice against invasive pneumococcal disease. We propose that c-di-GMP can be used as an effective broad spectrum immunomodulator molecule and vaccine adjuvant to prevent infectious diseases.

Analysis of safety and immunogenicity in adult and in newborn mice

We performed a series of preliminary experiments in neonatal and adult mice to evaluate the capacity of c-di-GMP to increase immunity to *Yersinia pestis* F1 and LcrV. As explained above, this molecule has been shown to enhance protection against bacterial pathogens. We hypothesized that it can serve as a potent stimulator of immune responses in young and neonatal mice. The use of the c-di-GMP as “vaccine adjuvant” is particularly appealing because it is not a biohazard and can be safely given by a mucosal route to young hosts. We starting by setting up a neonatal mouse model and performed a safety and preliminary immunogenicity analysis in adult and newborn mice.

Safety of c-di-GMP immunization in newborn mice

The first experiment was dose-response curve to determine the amount of c-di-GMP that is safe and non-reactogenic in 7-days old mice. It was necessary to establish this dose prior to the immunogenicity experiments. Four groups of newborn mice were randomized to receive increasing doses of c-di-GMP (i.e 50, 100 and 200 mnoles) by the intramuscular dose. A control group received PBS.

The site of inoculation and overall health status of the pups was carefully examined. Animals were euthanized at different time points after inoculation (24h, 48h, 72h or 14 days) and skin and muscle (from the site of injection) were collected for histological analyses.

We observed that the 50 nm dose did not cause visible adverse effects. Inflammation (redness, swelling) in the site of injection was observed in pups that received 100 nm. Inflammation and alopecia were observed in animals that received 200 nm of c-di-GMP (Figure 1). Both the inflammation and alopecia appeared within 2 days of dosing and were still evident 7 days after injection. Interestingly, doses up to 200 nm were well tolerated in adult mice. These findings highlight the importance of evaluating vaccine candidates in the appropriate animal models (and conditions) that can reflect all possible outcomes.

Clearly, newborn mice are more susceptibility to the immune stimulation induced by this adjuvant than adult mice, and a careful analysis is required to select the appropriate dose for use in young hosts. Based on published reports, a dose of 50 nm is still expected to have potent adjuvant properties(3).

Treatment	Observations	
CTRL only vehicle (NaCl)	→	
50 nmol c-di GMP	→	
200 nmol c-di GMP (positive control)	Inflammation and alopecia	

Figure1

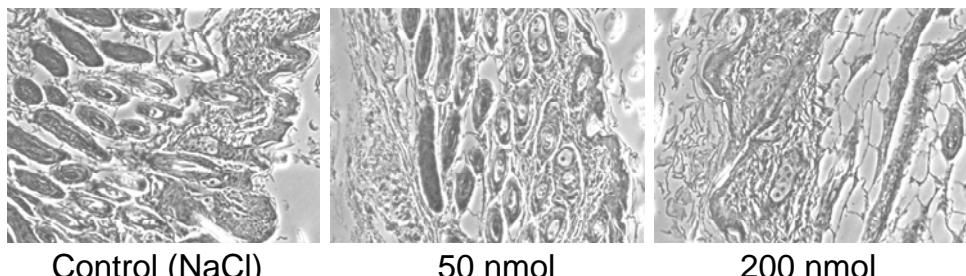


Figure 2

The histological analysis of skin collected 7 days after injection (Figure 2) revealed loss of tissue structure, edema, and infiltration of leukocytes that reached the subcutaneous tissue

Immunogenicity of *Y. pestis* F1 and LcrV in adult and newborn mice

An earlier experiment showed that adult mice responded with high levels of serum IgG antibodies to *Y. pestis* F1 and LcrV ($\sim > 1 \times 10^6$ EU/ml, measured by ELISA) when these recombinant proteins were mixed and administered by the intramuscular (i.m.) route in the presence of c-di-GMP (2 μ g of protein and 200 nm of c-di-GMP). A high frequency of F1- and LcrV-specific ASC was found in the bone marrow of immunized mice; these cells are believed to represent a pool of long-lived plasma cells ready to produce systemic antibodies. Significantly higher responses were seen in mice that received c-di-GMP compared with those immunized with F1 and LcrV in the presence of alum (data not shown).

The capacity of c-di-GMP to enhance neonatal immune responses to *Y. pestis* antigens F1 and LcrV was studied *in vivo*, in two experiments. In **Experiment 1**, newborn mice were immunized i.m. with 2 μ g of the F1/V subunit vaccine in the presence of c-di-GMP, using the two best tolerated doses identified in the safety analysis (i.e. 10 and 50 nmol). Control groups received F1/V alone or F1/V in the presence of alum (0.5%). A group of naïve mice was included as negative control. Figure 3 shows the LcrV and F1 serum IgG levels measured at different time points after immunization. High IgG responses to LcrV were detected in mice immunized with F1/V in the presence of alum, whereas negligible responses were produced when F1/V was given alone (in the absence of an adjuvant). No responses were detected in unvaccinated naïve mice.

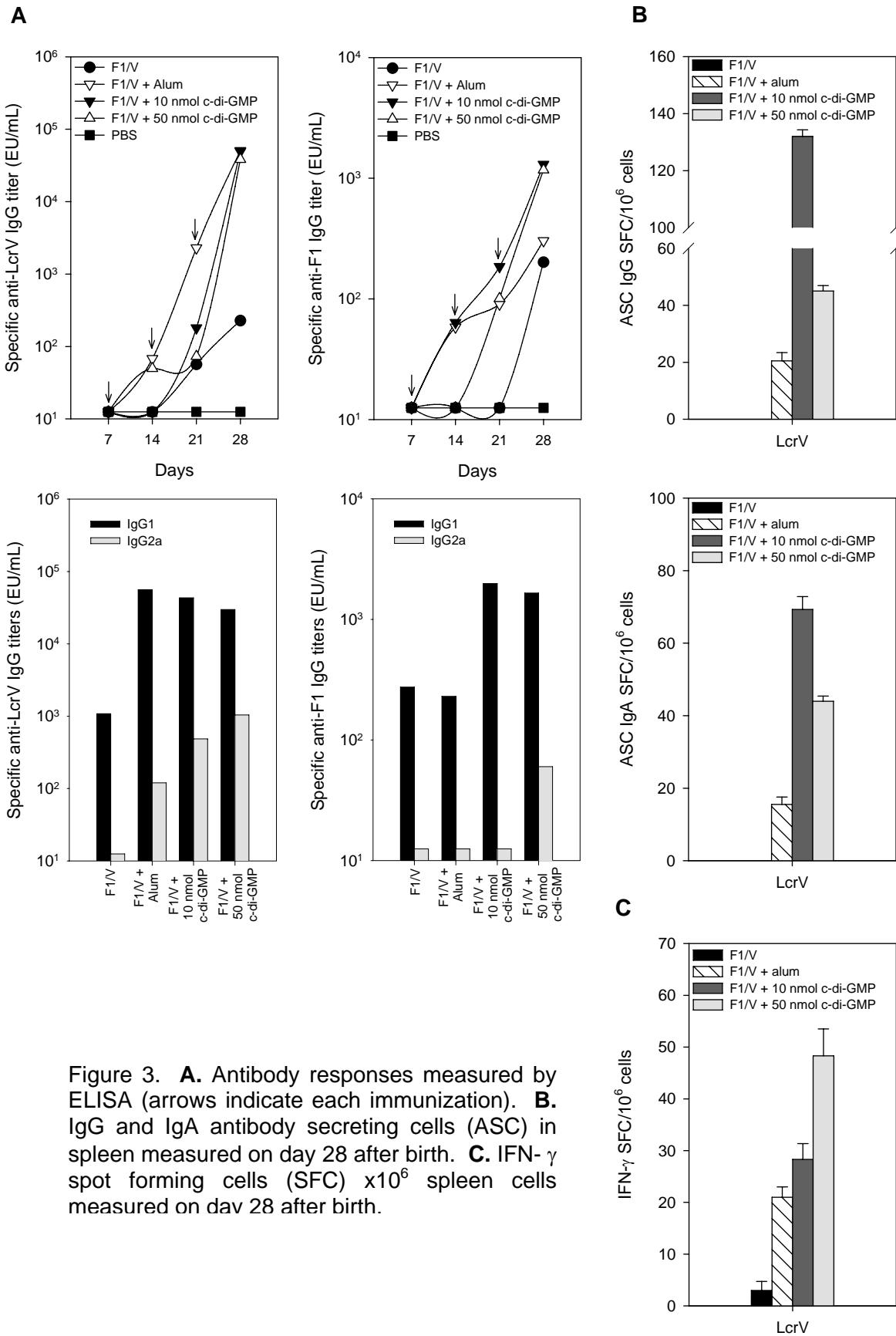


Figure 3. **A.** Antibody responses measured by ELISA (arrows indicate each immunization). **B.** IgG and IgA antibody secreting cells (ASC) in spleen measured on day 28 after birth. **C.** IFN- γ spot forming cells (SFC) $\times 10^6$ spleen cells measured on day 28 after birth.

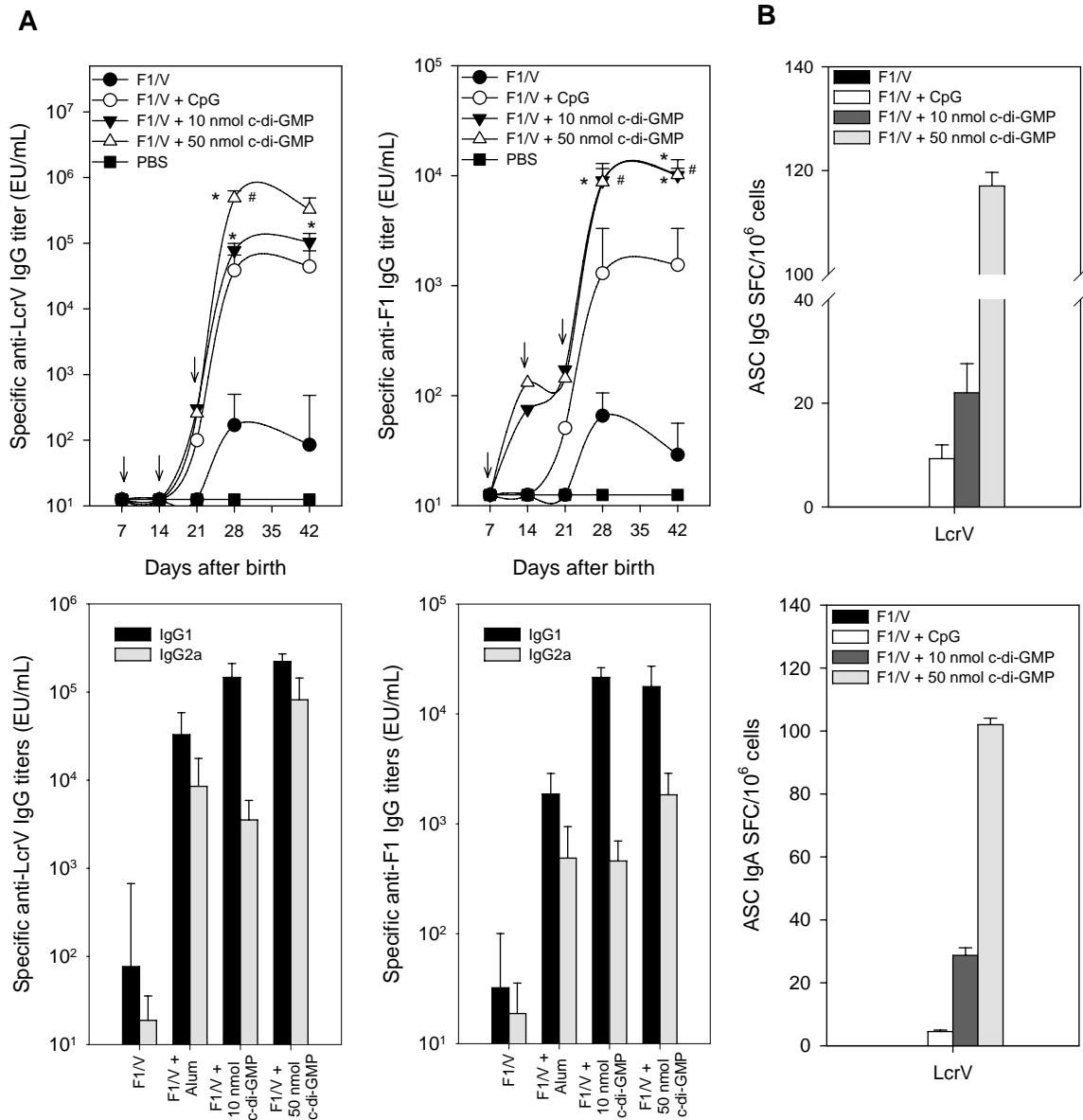


Figure 4. **A.** Antibody responses measured by ELISA. **B.** IgG and IgA ASC measured in spleen on day 28 after birth. * $p<0.05$ compared with the F1/V group and # $p<0.05$ compared with the F1/V + CpG group.

The highest F1-specific IgG responses were produced by newborns immunized with F1/V and c-di-GMP regardless of the dose of adjuvant used (10 or 50 nmol). Higher serum IgG2a levels were also produced when F1/V was given with c-di-GMP as compared with alum.

A further analysis of immune responses was performed focusing on LcrV as the main protective antigen and vaccine candidate; virulent *Y. pestis* F1(-) strains have been described and there is agreement in the literature that immune responses to LcrV are sufficient to protect against disease. Furthermore, in several measurements, responses to F1 were markedly lower than those raised against LcrV.

Increased frequencies of LcrV-specific IgG and IgA ASC were detected in the spleen of mice immunized as newborns with F1/V in the presence of c-di-GMP. These responses greatly surpassed those induced by F1/V administered with alum.

Newborns immunized with F1/LcrV and c-di-GMP also developed Th1-type cellular immunity. IFN- γ production, measured as frequency of IFN- γ spot forming cells (SFC) in spleen cells stimulated in vitro with LcrV, was significantly higher in animals immunized with F1/V in the presence of c-di-GMP, as compared with alum, and responses increased with higher amounts of c-di-GMP.

In **Experiment 2**, newborn mice were immunized i.n. with 10 μ g of F1/V and two dosage levels of c-di-GMP (10 and 50 nm). A positive control group received F1/V in the presence of CpG, a known Th1 type adjuvant (8). Naïve mice served as negative controls. Figure 4 shows the kinetics of LcrV and F1 serum IgG titers. For both antigens, the highest antibody responses were produced in mice that received the vaccine in the presence of c-di-GMP; the 50 nmol dose appeared to be more effective than the 10 nmol for antibody production. High levels of IgG2a were found in all groups. Systemic IgG and IgA LcrV-specific ASC as well as mucosal IgG and IgA ASC were detected in the group immunized with c-di-GMP. These responses surpassed (in most cases) those of mice immunized with F1/V and CpG.

Strong Th1 type responses were also induced. The frequency of IFN- γ -secreting cells in mice that received the vaccine with c-di-GMP surpassed those of mice immunized with F1/V and CpG. This is an important observation considering the CpG is a very potent Th1 type adjuvant.

Conclusions. Together these results suggest that c-di-GMP is a potent adjuvant for neonatal immunization, with the capacity to enhance systemic and mucosal antibody production and Th1 type responses during the neonatal period. In most instances, the responses stimulated in the presence of c-di-GMP surpassed those achieved using CpG.

Publications related to this grant

Karaolis, D. K. R., Means T.K., Yang, D., Yoshimura T., Muraille, E., Bowie A., Philpott, D., Schroeder, J., Hyodo, M., Hayakawa, Y., Brouillet E., Talbot B., and Malouin F. 2007. Bacterial c-di-GMP is an immunostimulatory molecule. *J. Immunol.* 178:2171-2181.

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2. *Antimicrob. Agents Chemother.* **49**:3109-3113.
2. **Brouillette, E., M. Hyodo, Y. Hayakawa, D. K. Karaolis, and F. Malouin.** 2005. 3',5'-cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection
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Bacterial c-di-GMP Is an Immunostimulatory Molecule¹

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Cyclic diguanylate (c-di-GMP) is a bacterial intracellular signaling molecule. We have shown that treatment with exogenous c-di-GMP inhibits *Staphylococcus aureus* infection in a mouse model. We now report that c-di-GMP is an immudulator and immunostimulatory molecule. Intramammary treatment of mice with c-di-GMP 12 and 6 h before *S. aureus* challenge gave a protective effect and a 10,000-fold reduction in CFUs in tissues ($p < 0.001$). Intramuscular vaccination of mice with c-di-GMP coinjected with *S. aureus* clumping factor A (ClfA) Ag produced serum with significantly higher anti-ClfA IgG Ab titers ($p < 0.001$) compared with ClfA alone. Intraperitoneal injection of mice with c-di-GMP activated monocyte and granulocyte recruitment. Human immature dendritic cells (DCs) cultured in the presence of c-di-GMP showed increased expression of costimulatory molecules CD80/CD86 and maturation marker CD83, increased MHC class II and cytokines and chemokines such as IL-12, IFN- γ , IL-8, MCP-1, IFN- γ -inducible protein 10, and RANTES, and altered expression of chemokine receptors including CCR1, CCR7, and CXCR4. c-di-GMP-matured DCs demonstrated enhanced T cell stimulatory activity. c-di-GMP activated p38 MAPK in human DCs and ERK phosphorylation in human macrophages. c-di-GMP is stable in human serum. We propose that cyclic dinucleotides like c-di-GMP can be used clinically in humans and animals as an immunomodulator, immune enhancer, immunotherapeutic, immunoprophylactic, or vaccine adjuvant. *The Journal of Immunology*, 2007, 178: 2171–2181.

Cyclic diguanylate (c-di-GMP)³ (3',5'-cyclic diguanylic acid or cGpGp) was first identified in *Acetobacter xylinum* (renamed *Gluconacetobacter xylinus*) as an intracellular allosteric activator regulating cellulose production (1–5). c-di-GMP has only been found in bacteria and not eukaryotes, suggesting that the molecule and c-di-GMP-mediated signaling is an exclusively bacterial trait (6–9). c-di-GMP is now increasingly being recognized as an ubiquitous intracellular signaling molecule in many microbial species. The level of intracellular

c-di-GMP in some species is believed to be regulated by the opposing effects of diguanylate cyclases for c-di-GMP synthesis from GTP and c-di-GMP phosphodiesterase for breakdown to 5'-GMP and each controlling the level of c-di-GMP in the cell. c-di-GMP has been shown to influence bacterial cell survival, cell differentiation, colonization and biofilm formation, as well as bacteria-host interactions (2, 3, 5–18). Although the role of intracellular c-di-GMP in bacteria is now recognized and is becoming an intense field of research, we have been studying the effect of this microbial molecule in the context of other biological systems and its potential clinical and therapeutic use for the prevention and inhibition of infection and disease (19–22).

We have previously demonstrated that exogenous c-di-GMP significantly reduces in vitro cell-cell interactions and biofilm formation of *Staphylococcus aureus*, including human methicillin-resistant *S. aureus* strains and animal clinical isolates (6, 19). We have also previously demonstrated in vivo clinical proof-of-concept efficacy that treatment with c-di-GMP (200 nmol) significantly attenuates *S. aureus* infection and reduces cell numbers (>10,000-fold; $p < 0.001$) in a mouse model of infection (21). In this later study, an unexpected finding was that c-di-GMP has no apparent inhibitory or bactericidal effect on *S. aureus* in vitro, yet in vivo it significantly reduced the colonization of the mammary glands by biofilm-forming *S. aureus* strains. The results of that study imply that c-di-GMP might have a biological effect on the in vivo environment and the host immune response. Until now, the effect of c-di-GMP on the host immune response has not been proposed or studied. In this study, we tested the hypothesis that c-di-GMP is not only an important microbial signaling molecule, but is also a novel immunostimulatory agent that can modulate the host immune response.

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Received for publication August 2, 2006. Accepted for publication November 17, 2006.

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¹ This work was supported by Grant MOP-57701 (to F.M.) from the Canadian Institute for Health Research. D.K.R.K. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences. E.M. is a Research Associate from the Fonds National de la Recherche Scientifique.

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³ Abbreviations used in this paper: c-di-GMP, cyclic diguanylate; DC, dendritic cell; PDC, plasmacytoid cell; ClfA, clumping factor A; ODN, oligodeoxynucleotide; QPCR, quantitative PCR; MFI, mean fluorescent intensity; Nod, nucleotide-binding oligomerization domain; HEK, human embryonic kidney.

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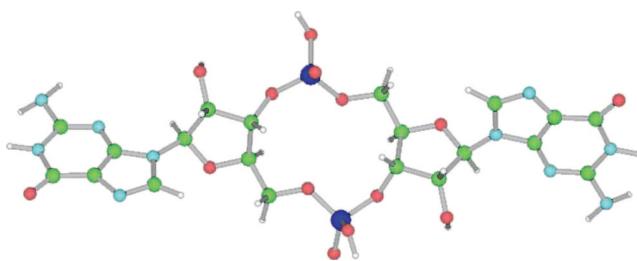


FIGURE 1. Structure of c-di-GMP. Ball and stick model of c-di-GMP. Light green ball, carbon; light blue ball, nitrogen; red ball, oxygen; dark blue ball, phosphorus; and white ball, hydrogen.

Materials and Methods

c-di-GMP and chemicals used

The c-di-GMP (Fig. 1) used in these studies was synthesized and prepared as previously described (19–24). Zymosan A (*Saccharomyces cerevisiae*), poly(I:C) (dsRNA), and LPS (*Escherichia coli* K12) were purchased from Sigma-Aldrich. Synthetic lacylated lipopeptide *N*-palmitoyl-cysteine-serine-lysine (PAM₃CSK₄) was purchased from Bachem. Flagellin was purified from *Salmonella typhimurium*. R848 (resiquimod) was purchased from GL Synthesis. Oligonucleotide CpG-A (GGTGCATCGATGCAGGG GGG) used to stimulate monocyte-derived dendritic cells (DCs) was purchased from InvivoGen. The CpG-A used in activating plasmacytoid DCs (pDCs) was obtained from Invitrogen Life Technologies and is called oligodeoxynucleotide (ODN) 2216 (25).

LPS endotoxin (*Limulus amebocyte lysate* assay) test

A dose of 500 μ M c-di-GMP was tested for the presence of LPS endotoxin using the *Limulus amebocyte lysate* assay (Charles River Laboratories).

Pretreatment prophylactic studies in the mouse mastitis model

The well-characterized mouse model of *S. aureus*-induced mastitis (for review, see Ref. 26) was used to evaluate the capacity of c-di-GMP to act as a prophylactic agent. The protocol used here is similar to that described in a previous study (21), except that the c-di-GMP molecule was administered before infection of mammary glands, instead of after, to evaluate its prophylactic capacity. Briefly, 12 and 6 h before bacterial inoculation, saline or 50 or 200 nmol of c-di-GMP was administered into the fourth pair of abdominal mammary glands of lactating CD-1 mice. Administration of 50 and 200 nmol of c-di-GMP corresponded to a dose of 1.0 and 4.1 mg of compound per kg of body weight, respectively, considering an average weight of 35 g for a mouse. For inoculation, 100 CFU of *S. aureus* Newbould 305 (ATCC 29740) were injected into each gland and the colonization was allowed for 10 h. Raw bacterial CFU counts obtained after plating serial logarithmic dilutions of mammary gland homogenates were transformed in base 10 logarithm values. The experiment was repeated and data combined for a total of nine mice (18 glands) per group. Since values passed the normality test, bacterial counts were analyzed for statistical significance using the one-way ANOVA test combined with the Tukey posttest (GraphPad Instat, version 3.06). The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke approved these experiments and were conducted following the guidelines of the Canadian Council on Animal Care.

Vaccine adjuvant studies in mice

To evaluate the capacity of c-di-GMP to increase Ab production in the mouse, the cyclic dinucleotide was used as adjuvant for vaccination with *S. aureus* clumping factor A (ClfA) recombinant protein (a surface adhesion protein of *S. aureus* binding fibrinogen), as previously done for ClfA alone without adjuvant (27). Two groups of eight CD-1 female mice were vaccinated i.m. with 100 μ l of saline containing 25 μ g of ClfA. For one of the groups, the solution injected also contained 200 nmol of c-di-GMP. A volume of 50 μ l was then injected into both quadriceps. This procedure was repeated 2 wk after, for a second injection. Twelve days later, i.e., 26 days after the initial injections, blood samples were taken and incubated at 35°C for 1 h to allow coagulation and then centrifuged at 13,000 \times g for 10 min at 4°C. Sera were harvested and kept at -20°C until tested. The institutional ethics committee on animal experimentation of the Faculté des Sciences of Université de Sherbrooke approved these experiments and were conducted according to the guidelines of the Canadian Council on Animal Care.

Ab assays

Total IgGs. ELISA were used to determine the presence of IgG Abs against the ClfA Ag in the mouse serum as previously described (27). Polystyrene Maxisorp 96-well plates (Nalge Nunc International) were coated for 2 h with 50 μ l of recombinant ClfA (domain A) protein at a concentration of 10 μ g/ml in carbonate/bicarbonate buffer at pH 9.6. Following saturation of the wells with a solution of powdered milk in PBS (5% w/v), overnight at 4°C, 4-fold dilutions of sera (1/1000–1/64000) were added and incubated for 2 h at 35°C and then overnight at 4°C. Biotinylated anti-mouse IgG (1/1000) was added and incubated for 2 h at 35°C. After 1 h of incubation with streptavidin-HRP (Amersham Pharmacia Biotech) diluted 1/500, 100 μ l of Sure Blue Tetramethylbenzidine Peroxidase Substrate (Kirkegaard & Perry Laboratories) was added. The enzyme reaction was stopped by the addition of 50 μ l of 1 N HCl after 6 min of incubation. Between each step, three washes with PBS-0.05% Tween 20 were conducted. The OD was read on a plate reader (Bio-Tek Instruments) at 450 nm. Each sample was tested in triplicate and the OD of the negative control wells that did not contain Ag was subtracted from the ClfA-coated test wells.

IgG isotypes. ELISA were conducted as for the total IgG assay but the secondary Ab was either mouse anti-IgG1-HRP or mouse anti-IgG2a-HRP (BD Pharmingen), and 4-fold dilutions of sera from 1/250 to 1/16,000 were assayed. Quantitation of mouse Abs was achieved as for the total IgG assay but the development of coloration was allowed for 10 min. Each sample was tested in triplicate and the OD of the negative control wells that did not contain Ag was subtracted from the ClfA-coated test wells. For the statistical analysis of ELISA results, the OD data for mice vaccinated with the c-di-GMP molecule as adjuvant were matched up to the corresponding data (isotype and dilution) where mice were injected with saline instead of c-di-GMP. One-way ANOVA was used with the Bonferroni post hoc test to account for multiple comparisons (GraphPad InStat software, version 3.06). For all isotypes and dilutions analyzed, statistical significance was found to be $p < 0.001$ between the groups.

c-di-GMP activates monocyte and granulocyte recruitment *in vivo*

C57BL/6 mice were injected with a volume of 500 μ l containing 200 nmol of c-di-GMP. Cervical dislocation was performed after 12 h, after which with 10 ml of cold PBS was injected and the fluid was withdrawn and centrifuged at 1400 rpm at 4°C. Fluid was incubated in PBS/BSA/azide plus 24G2 (anti-Fc) for 15 min at 4°C, washed in PBS, then stained and analyzed by FACS analysis (PBS/BSA/azide, 30 min 4°C). Cells were stained with directly coupled fluorescent mAb combinations in 200 ml of FACS buffer and further collected on a FACSCalibur cytofluorometer (BD Biosciences). We purchased the following mAbs: fluorescein (FITC)-coupled M1/70 (anti-CD11b; BD Pharmingen), PE-coupled 1A8 (anti-LY-6G; BD Pharmingen), 7/4 (Caltag Laboratories), and allophycocyanin-coupled F4/80 (Caltag Laboratories). Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Murine DC isolation

Murine splenic DCs were isolated from spleens of C57BL/6 mice. Briefly, spleens were dissected into small pieces and incubated at 37°C in complete RPMI 1640. Cell suspension was obtained by vigorous pipetting and passage through a 70- μ m nylon mesh filter. After RBC lysis, CD11c⁺ DCs were isolated using CD11c microbeads according to the manufacturer's instructions (Miltenyi Biotec). The cells were >95% CD11c⁺ as measured by FACS analysis.

Preparation and treatment of human monocyte DCs

Cytokine, chemokine, and chemokine receptor analysis. Buffy coats were obtained from healthy volunteers and fractionated over Histopaque 1077. The PBMC layer was recovered and erythrocyte depleted by incubation in RBC lysis buffer for 5 min at room temperature. PBMCs were cultured in complete medium (RPMI 1640, 1% L-glutamine, 1% penicillin/streptomycin, and 10% low endotoxin FCS) for 2 h in T75 flasks (Corning). Following incubation, nonadherent cells were removed by three washes with 1× PBS (Invitrogen Life Technologies). The remaining adherent cells were then cultured in complete medium supplemented with GM-CSF and IL-4 (50 ng/ml each). Human and murine IL-4 and GM-CSF were purchased from PeproTech. On days 2 and 4, the DC cultures received an additional dose of GM-CSF and IL-4 (50 ng/ml each). On day 5, nonadherent DCs were harvested by gentle pipetting, counted, and plated in fresh medium containing GM-CSF and IL-4 (50 ng/ml each). On day 6, some DCs were matured by addition of 100 ng/ml LPS or 10–400 μ M c-di-GMP for 1–24 h.

p38 MAPK signaling studies. Human PBMCs were isolated by Ficoll density gradient centrifugation from leukopacks supplied by the Department of Transfusion Medicine (Clinical Center, National Institute of Health, Bethesda, MD). Monocytes were purified (>95%) from human PBMCs with a MACS CD14 monocyte isolation kit (Miltenyi Biotec) according to the manufacturer's instruction. DCs were generated as described previously (28). In brief, DCs were generated by incubating purified monocytes at 2×10^5 /ml in RPMI 1640 containing 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 ng/ml recombinant human GM-CSF (PeproTech), and 50 ng/ml recombinant human IL-4 (PeproTech) at 37°C in a CO₂ (5%) incubator for 7 days. On days 3 and 5 of the incubation, half of the culture medium was replaced with prewarmed (37°C) fresh GM-CSF- and IL-4-containing medium. On day 7, DCs were collected, washed three times with PBS, and serum-starved for 8 h by incubating in serum-free RPMI 1640 containing GM-CSF and IL-4 (2×10^5 /ml) at 37°C in a CO₂ (5%) incubator. Subsequently, DCs (2×10^6 /sample) were incubated at 37°C in the absence or presence of c-di-GMP or GMP (Sigma-Aldrich) at concentrations specified for 5 or 30 min. At the end of the incubation, the stimulation was immediately stopped by the addition of large amounts of ice-cold PBS (10-fold). The cells were centrifuged at 1500 $\times g$ for 5 min at 4°C, washed with cold PBS, and lysed by SDS sample buffer (62.5 mM Tris-HCl (pH 6.8) at 25°C, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue). The lysates were sonicated for 10 s to shear DNA, boiled for 5 min, and cooled down on ice.

Preparation and treatment of human pDCs

Venipuncture was performed on consenting adults using approved protocols. pDCs were prepared, as previously described (29), using a two-step procedure involving Percoll density centrifugation followed with selection for blood DC Ag 4-positive cells (Miltenyi Biotec). The capacity of c-di-GMP to induce IFN- α from these cells was assessed using cell culture and ELISA protocols also previously described (29) and was simultaneously compared with the IFN- α produced by pDCs in response to cells stimulated with CpG oligodeoxynucleotide (ODN) 2216 (25).

Preparation and treatment of human M-CSF-induced monocyte-derived macrophages

Preparation of macrophages was performed as previously described (30). Briefly, fully differentiated macrophages from human PBMCs were isolated from leukapheresis preparations obtained by the Blood Bank, Clinical Center, National Institutes of Health. The leukocyte-rich preparation was overlaid on Accu-Prep in 50-ml tubes and the tubes were centrifuged at 800 $\times g$ for 20 min at room temperature. PBMC fractions were collected, washed once with PBS at room temperature and twice with complete medium at 4°C, and resuspended in the same medium. Monocytes were further purified by using iso-osmotic Percoll gradient (Amersham Biosciences). At this stage, the purity of monocytes was higher than 90%. One and half million cells were placed on a 12-well plate in 1.5 ml of RPMI1640 containing 50 ng/ml human M-CSF. On day 4, 1 ml of the medium was replaced with 1 ml of fresh medium containing 50 ng of human M-CSF. On day 7, nonadherent cells were washed out and adherent cells were used as macrophages.

Quantitative PCR (QPCR)

Total RNA was extracted using the RNeasy kit according to the manufacturer's protocol (Qiagen). Briefly, after DNase I (Invitrogen Life Technologies) treatment, 1 μ g of total RNA from each sample was used as template for the reverse transcription reaction. Fifty microliters of cDNA was synthesized using oligo(dT)₁₅, random hexamers, and multiscribe reverse transcriptase (Applied Biosystems). All samples were reverse transcribed under the same conditions (25°C for 10 min, 48°C for 30 min) and from the same reverse transcription master mix to minimize differences in reverse transcription efficiency. All oligonucleotide primers for QPCR were designed using Primer Express software 1.0 (PE Biosystems) and synthesized by Invitrogen Life Technologies. The 25- μ l QPCR contains 2 μ l of cDNA, 12.5 μ l of 2 \times SYBR Green master mix (Stratagene), and 250 nmol of sense and antisense primer. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Emitted fluorescence for each reaction was measured during the annealing/extension phase, and amplification plots were analyzed using the MX4000 software version 3.0 (Stratagene). Quantity values (i.e., copies) for gene expression were generated by comparison of the fluorescence generated by each sample with standard curves of known quantities. Next, the calculated number of copies was divided by the number of copies of GAPDH.

Quantitation of murine cytokines by ELISA

Murine IL-8 and TNF protein levels in the DC culture supernatant were measured by sandwich ELISA (R&D Systems).

T cell proliferation assay

Immature DCs and c-di-GMP-treated DCs were washed three times, diluted in fresh complete medium, and used as allogenic stimulators. Cells were seeded in 96-well round-bottom culture plates with APC serial dilutions, ranging from 20,000 to 400 DCs/well, and were mixed along with freshly purified CD3⁺ T cells (100,000/well). After 5 days of incubation, cells were pulsed with 1 μ Ci of [³H]thymidine/well for 18 h and were harvested on filter paper. Proliferative responses were measured as [³H]thymidine incorporation by an automatic beta counter. Tests were performed in triplicates, and results were expressed as the mean cpm.

Flow cytometry

Surface expression of various markers was assessed using CellQuest analysis software on a FACSCalibur (BD Biosciences) flow cytometer. Surface expression was determined using the following FITC- and PE-conjugated Abs: CD86-FITC, CD83-FITC, HLA-DR-FITC (BD Pharmingen). The isotype control Abs were used accordingly in all experiments and were purchased from BD Pharmingen. Human DCs were incubated in 1% human AB serum/PBS and incubated with rat anti-CD16/CD32 (BD Pharmingen) to block nonspecific binding.

SDS-PAGE and Western blot

DCs. The lysates were loaded (15 μ l/lane) and separated on a 4–12% NuPAGE Bis-Tris gel (Invitrogen Life Technologies) using 1 \times NuPAGE MES SDS running buffer (Invitrogen Life Technologies) as the electrode buffer. SeeBlue Plus2 (Invitrogen Life Technologies) was used as molecular size marker. After electrophoresis, proteins in the gel were electrotransferred (25 V constant for 1 h) onto a piece of Immobilon membrane (Millipore) using 1 \times NuPAGE transfer buffer (Invitrogen Life Technologies). The membrane was sequentially washed, blocked for 1 h at room temperature, washed, and incubated at 4°C overnight in the presence of a 1/1000 dilution of rabbit anti-phospho-p38 MAPK Ab (Cell Signaling Technology). On the next day, the membrane was washed and incubated with a 1/2000 dilution of HPR-conjugated anti-rabbit IgG (Cell Signaling Technology) for 1 h. After washing, and incubation with a working solution of ECL Plus Western Blotting Detection System (Amersham Biosciences) for 5 min at room temperature, the membrane was exposed to a piece of BioMax x-ray film (Kodak). The x-ray film was developed using an automatic processor (Kodak X-OMAT 200A). The same membrane was stripped and probed for p38 MAPK protein essentially in the same manner except rabbit anti-p38 Ab (Cell Signaling Technology) as the primary Ab was used.

Macrophages. Western blot analysis of macrophages was performed as previously described (31). Briefly, prepared macrophages were starved in RPMI 1640 without FCS for 10 h. Cells were rinsed three times with PBS and treated with cyclic dinucleotide for various times. Cells were lysed on ice for 20 min in a buffer containing 50 mM NaCl, 20 mM Tris-HCl, 50 mM sodium fluoride, 30 mM Na₄P₂O₇, 5 mM EGTA, 3 mM sodium orthovanadate, 1% Triton X-100, 1 mg/ml leupeptin, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml pepstatin A, and 100 μ M sodium orthovanadate pre-treated with H₂O₂. The lysates were spun in a microcentrifuge for 20 min and the supernatants were collected. Proteins were analyzed on 12% polyacrylamide gels by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1 h in a semidry system. The membranes were incubated with an Ab against either phosphorylated or nonphosphorylated p38 MAPK or ERK, followed by an appropriate secondary Ab coupled with HRP. Peroxidase activity was visualized using LumiGLO (Cell Signaling Technology).

TLR and nucleotide-binding oligomerization domain (Nod) studies

For TLR studies, the human embryonic kidney (HEK) cell line HEK 293(CRL-1573) from American Type Culture Collection was used. HEK 293 cell clones stably expressing TLRs were generated by transfection of TLR cDNA, G418 antibiotic selection, and FACS sorting. HEK 293 cells were cultured in DMEM supplemented with 10% FBS. Cells were plated in 24-well tissue culture plates (1 \times 10⁵ cells/well) and maintained in the above medium for 24 h. The following day, cells were either left untreated or incubated with the indicated amount of TLR ligand. After the 3-h treatment period, cells were harvested for total RNA using the Qiagen RNeasy

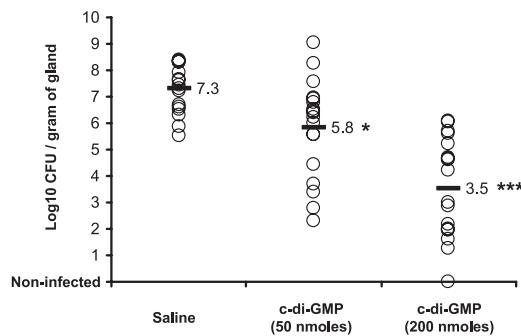


FIGURE 2. Pretreatment protective effect of c-di-GMP. c-di-GMP was administered 12 and 6 h before infection with *S. aureus*. Lactating mice were infected by intramammary inoculation and the infection was allowed for 10 h before mammary glands were harvested for bacterial CFU determination. Each circle on the graph corresponds to the number of CFU per gram of gland for an individual gland. Mean values are indicated and show that prophylaxis with c-di-GMP significantly reduced the level of *S. aureus* colonization in a dose-dependent manner (*, $p < 0.05$; ***, $p < 0.001$).

kit per the manufacturer's protocol. The total RNA was converted to cDNA and QPCR was performed to determine the number of copies of IL-8. For Nod studies, experiments examining the synergistic activation of NF- κ B by c-di-GMP in cells overexpressing Nod1 or Nod2 were conducted as previously described (32). Briefly, HEK 293 T cells were transfected overnight with 30 ng of Nod1 or Nod2 plus 75 ng of Igk luciferase reporter plasmid. Ten nanograms of a constitutive *Renilla* luciferase reporter was also transfected into cells to adjust for transfection efficiency. At the same time, 200 μ M c-di-GMP, *N*-acetyl muramyl-L-Ala- γ -D-Glu-meso-DAP (Nod1 ligand) or muramyl dipeptide (Nod2 ligand) were added and the synergistic NF- κ B-dependent luciferase activation was then measured following 24 h of coincubation. NF- κ B-dependent luciferase assays were based on two independent experiments performed in duplicate.

Stability of c-di-GMP in human serum

Commercially supplied pooled human serum (100 μ l; Cambrex) was diluted in ion-exchanged water. An aliquot of the resulting human serum solution was subjected to HPLC analysis (conditions: COSMOSIL 5C18-AR-II column (4.6 \times 200 mm); buffer A; buffer B, 80% acetonitrile in water; gradient, 0–10 min A (100%), 10–60 min A:B = 100:0 to A:B = 40:60 in 50 min; detection 254 nm, flow rate, 1.0 ml/min, temperature 40°C). Separately, a 100 μ M c-di-GMP solution in human serum was prepared by adding 100 μ l of a 500 μ M c-di-GMP aqueous solution to a

mixture of 390 μ l of human serum and 10 μ l of 40 mM 2-methylbenzimidazole (an internal standard for estimating the extent of decomposition of c-di-GMP) in methanol. An aliquot of the test solution was diluted immediately after the preparation solution and then heated at 100°C for 5 min to quench activity of the enzymes. The resulting test sample was subjected to HPLC analysis after a 24-h treatment. Peaks that appeared in both analyses were compared. Results are based on three independent experiments.

Results

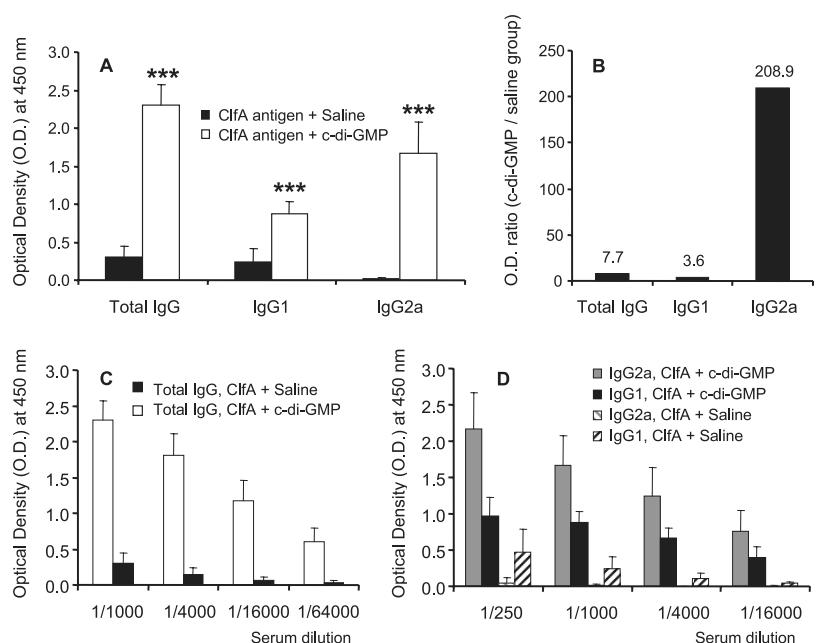
Prophylactic pretreatment of mice with c-di-GMP inhibits infection in vivo

We previously reported that intramammary injections of c-di-GMP significantly decreased the colonization of the mouse mammary glands by *S. aureus* when the cyclic dinucleotide was administered twice, at the time of the bacterial challenge, and at 4 h after inoculation (21). In this study, we further investigated whether c-di-GMP had any prophylactic effect when provided hours before the bacterial inoculation of the mouse mammary glands. We first performed an in vivo study to address the following questions: is c-di-GMP stable in tissues at the site of infection and will c-di-GMP stimulate a host immune response? In this study, c-di-GMP was given –12 h and –6 h before bacterial challenge. Even though the mammary glands were full of milk (~250 μ l) at the time of injection with infectious bacteria, Fig. 2 shows that pretreatment with c-di-GMP 12 and 6 h before bacterial challenge produces a significant prophylactic effect with a 1.5 and 3.8 log (~10,000-fold) reduction of the mean bacterial CFU in tissues using a 50- and 200-nmol dose, respectively, compared with the untreated control ($p < 0.05$ and $p < 0.001$, respectively). Had c-di-GMP only inhibited biofilm formation, as previously shown in our in vitro (19) and in vivo models (21), we would not have expected to find such dramatically reduced numbers of bacterial cells between pretreated and nontreated mice. These results suggest that c-di-GMP might stimulate the innate immune response.

c-di-GMP has adjuvant properties

Based on our previous in vivo results suggesting that c-di-GMP stimulates the host response and inhibits infection, we performed additional experiments to address basic fundamental questions on the effects of c-di-GMP on the host immune response. To evaluate the possibility that c-di-GMP could also act as an adjuvant, the

FIGURE 3. Adjuvant effects of c-di-GMP. *A*, Abs in serum of mice vaccinated with the ClfA Ag, with or without concomitant administration of c-di-GMP, as detected by ELISA. The histogram shows optical densities for serum samples that were diluted 1/1000 (***, $p < 0.001$). *B*, OD ratios (c-di-GMP/saline group) for results shown in *A*. *C*, Total IgG and *D*, IgG2a and IgG1 isotypes for serum samples diluted as shown. *C* and *D*, For each particular type of Ab assay and dilution tested, OD results for mice injected with c-di-GMP vs saline are statistically different from each other ($p < 0.001$), as found by one-way ANOVA used in conjunction with the Bonferroni post hoc test.



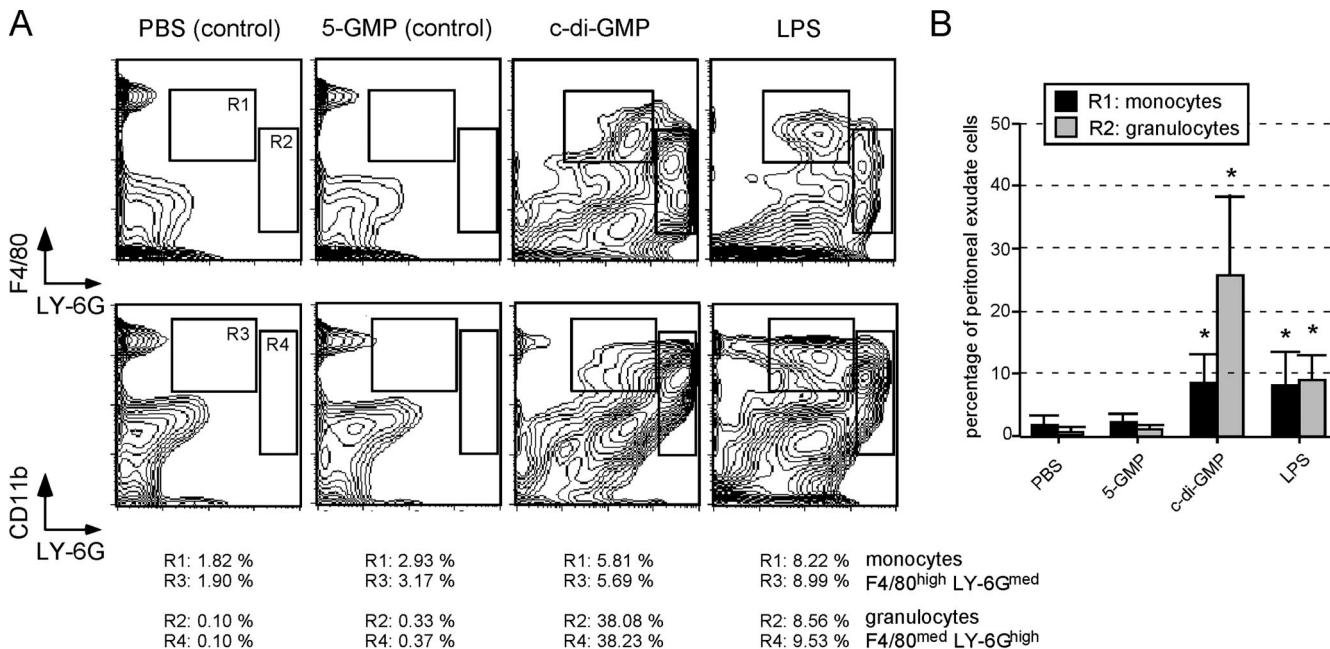


FIGURE 4. Intraperitoneal injection of c-di-GMP activates monocytes and granulocyte recruitment. Peritoneal fluid was analyzed by FACS analysis 18 h after injection of 200 nmol of c-di-GMP or 50 μ g of LPS. *A*, Contour plot of F4/80/LY-6G and CD11b/LY-6G showing recruitment of F4/80^{high} LY-6G^{med} (monocytes) and F4/80^{med} LY-6G^{high} (granulocytes). *B*, Histogram showing recruitment of macrophages and granulocytes. These results are representative of three independent experiments with three animals per group. Difference in relation to controls are denoted by an asterisk for $p < 0.05$.

cyclic dinucleotide was coinjected into mice with the recombinant ClfA Ag, a surface adhesion protein of *S. aureus* (33). Following vaccination with two i.m. injections of a mixture of ClfA and c-di-GMP, serum samples analyzed 12 days after the last injection (i.e., day 26) showed significantly higher anti-ClfA IgG Ab titers ($p < 0.001$) compared with injections of ClfA alone (Fig. 3). ELISA showed optical densities for total IgG and for the IgG1 and IgG2a isotypes that were above or around 0.5 at serum dilutions of 1/64,000 and 1/16,000, respectively, for the c-di-GMP-treated group, whereas OD densities were below 0.5 for serum dilutions $>1/1,000$ for the group vaccinated with ClfA without c-di-GMP (Fig. 3D). In Fig. 3, *C* and *D*, for each particular type of Ab assay and dilution tested, OD results for mice injected with c-di-GMP vs saline are statistically different from each other ($p < 0.001$), as found by one-way ANOVA used in conjunction with the Bonferroni post hoc test. Thus, in terms of OD values at a serum dilution of 1/1000, c-di-GMP coinjection increased total IgG by 7.7 times, IgG1 by 3.6 times, and IgG2a by 208.9 times (Fig. 3B). The relative increase in production of IgG2a in the presence of c-di-GMP indicates activation of the Th1 pathway. Although we cannot confirm Ag-specific cytokine production without testing, the other results suggest this. In any case, the data clearly demonstrate that c-di-GMP improves Ab production.

c-di-GMP activates monocytes and granulocyte recruitment in vivo

In support of the immunostimulatory activity mediated by c-di-GMP, studies in naive mice injected i.p. with c-di-GMP show that c-di-GMP induced the recruitment of F4/80^{high} LY-6G^{med} (monocytes) and F4/80^{med} LY-6G^{high} (granulocytes). Fig. 4 shows the results of studies in which FACS analysis using F4/80, LY-6G, and CD11b Ab was used to identify monocytes and granulocytes in peritoneal lavage at 18 h following i.p. injection of 200 nmol of c-di-GMP and 50 μ g of LPS (positive control). The in vivo recruitment of monocytes and granulocytes into the peritoneal cavity in response to c-di-GMP is likely the outcome of local

induction of certain chemokines (such as MCP-1) and the enhancement of adhesion molecules on either monocytes or endothelial cells.

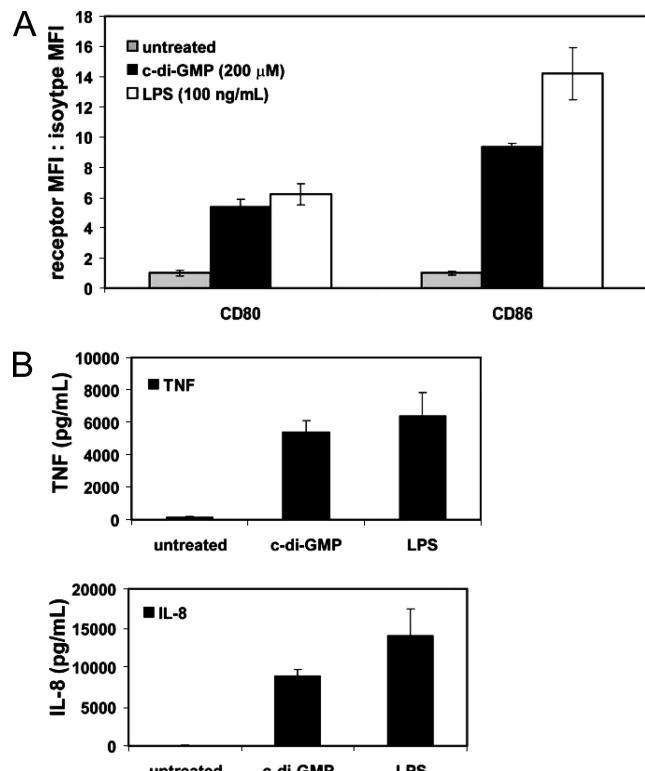


FIGURE 5. c-di-GMP activates murine DCs. *A*, Murine splenic DCs were stimulated with 200 μ M c-di-GMP for 24 h and stained with PE- or FITC-conjugated Abs specific for CD80 and CD86. *B*, The concentrations of the cytokines IL-8 and TNF in the above supernatants were measured by ELISA. The results shown are representative of three similar experiments.

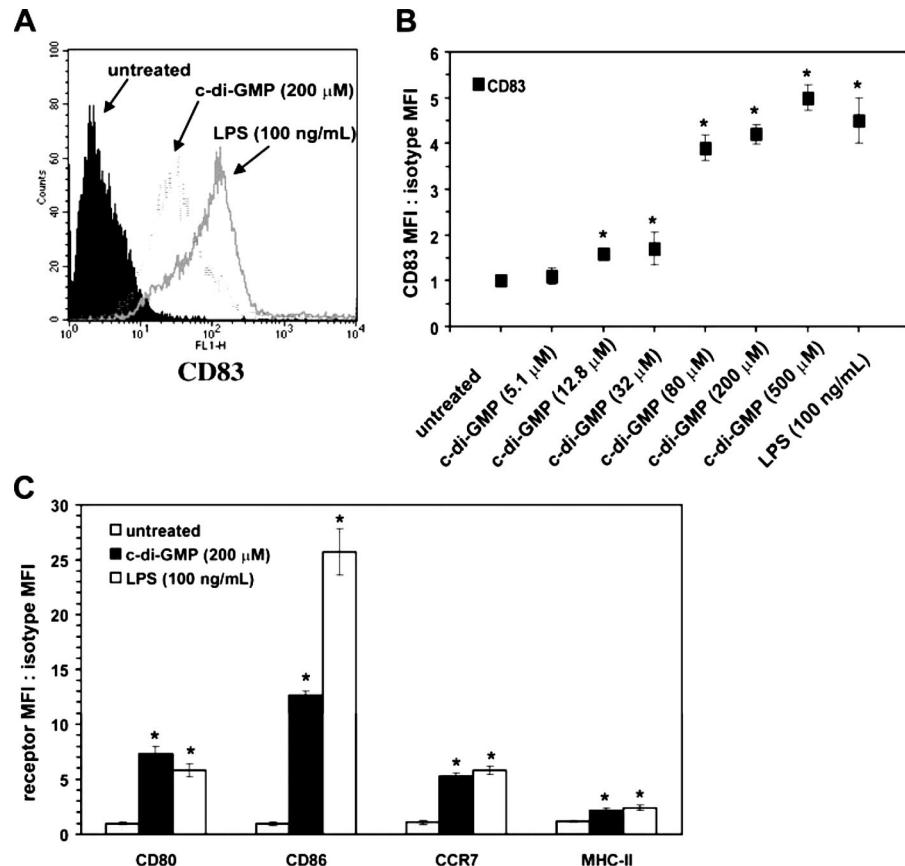


FIGURE 6. c-di-GMP activates the maturation of human DCs. *A*, Human immature DCs were stimulated with 200 μ M c-di-GMP for 24 h. Cells were stained with PE-conjugated Ab specific for CD83 or isotype control and were examined by flow cytometry. *B*, Dose response of DCs to c-di-GMP. This is a representative histogram of CD83 staining of LPS- and c-di-GMP-treated immature DCs. *C*, Immature DCs were treated with 200 μ M c-di-GMP for 24 h and stained with PE- or FITC-conjugated Abs specific for CD80, CD86, CCR7, or MHC class II. LPS stimulation of immature DCs was used as a positive control in all experiments. The bar graph represents the ratio of the MFI of the marker specific Abs to the MFI of the isotype control. The results shown are from a single experiment with a single donor and are representative of three similar experiments. *, $p < 0.01$, as determined by Student's *t* test and error bars indicate SD of triplicate measurements.

c-diGMP matures murine DCs

We decided to test whether c-di-GMP could also induce maturation of DCs. DCs are central in the immune response as they sense infection and respond appropriately to induce T cell immunity and promote a Th1 immune response. Maturation of DCs and their ability to become potent APC is critical for initiation of immune responses.

Primary murine CD11c $^{+}$ DCs were isolated from the spleen of C57BL/6 mice and treated with 200 μ M c-di-GMP for 24 h. We observed an increase in the surface expression of the costimulatory molecules CD80 and CD86 (Fig. 5). LPS-treated DCs were used as a positive control in this study and also induced a mature phenotype. To test whether c-di-GMP could induce the production of proinflammatory cytokines and chemokines, we analyzed the supernatants of c-di-GMP-treated DCs. We observed an increase in TNF and IL-8 protein in the supernatant of c-di-GMP-treated murine DCs (Fig. 5).

c-di-GMP matures human DCs

We decided to also test whether c-di-GMP could induce the maturation and activation of human DCs. We used an in vitro culture system for the production of human monocyte-derived DCs in which adherent human monocyte-derived DCs prepared from adherent PBMCs cultured in IL-4 and GM-CSF for 5 days were used in these experiments. Immature DCs (defined by FACS analysis in which DCs express no CD14 or CD83, but high levels of CD11c) were treated for 24 h with 5–500 μ M c-di-GMP. Fig. 6A shows a representative histogram comparing the increased expression of CD83 in DCs treated with 200 μ M c-di-GMP (net mean fluorescent intensity (MFI) = 63.34) compared with that of untreated cells (net MFI = 4.89) or cells treated with LPS (net MFI = 141.22) used as negative and positive controls, respec-

tively. A dose-dependent increase in surface CD83 expression was observed on cells treated with a wide range of c-di-GMP concentrations, with significant increases beginning at 32 μ M and spiking at 500 μ M, the highest concentration tested (Fig. 6B). We also observed an increase in the expression of other maturation markers (CD80, CD86, CCR7, and MHC class II) on DCs treated with 200 μ M c-di-GMP (Fig. 6C).

Cytokine, chemokine, and chemokine receptor expression in c-di-GMP-induced DC maturation

To examine the potential of c-di-GMP-treated DCs in activating and/or recruiting other effector cells to sites of infection, we measured whether cytokine, chemokine, and chemokine receptor expression is altered in DCs treated with c-di-GMP. c-di-GMP stimulated mRNA expression of the regulatory cytokine IFN- α , the proinflammatory cytokines IL-1 β and TNF, as well as the Th1 cytokines IFN- γ and IL-12p40 (Fig. 7A). We found no evidence for the induction of Th2 cytokines (IL-4, IL-5, IL-10, IL-13) by c-di-GMP-treated human DCs. To demonstrate that increases in IL-8 and IFN- γ -inducible protein 10 mRNA levels were accompanied by protein production, ELISA was performed on the supernatants of DCs stimulated with c-di-GMP stimulated for 24 h. These data were consistent with the mRNA results (data not shown). Fig. 7B shows that treatment with c-di-GMP caused an increased mRNA expression of several chemokines in DCs, notably IL-8/CXCL8, monokine induced by IFN- γ (MIG)/CXCL9, IFN- γ -inducible protein 10/CXCL10, IFN-inducible T cell α chemotactant/CXCL11, MCP-1/CCL2, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5.

The migration of DCs from the periphery, where they encounter and take up foreign Ag, to the T cell area of the lymph nodes is an important aspect of immunity. To determine the potential c-di-GMP

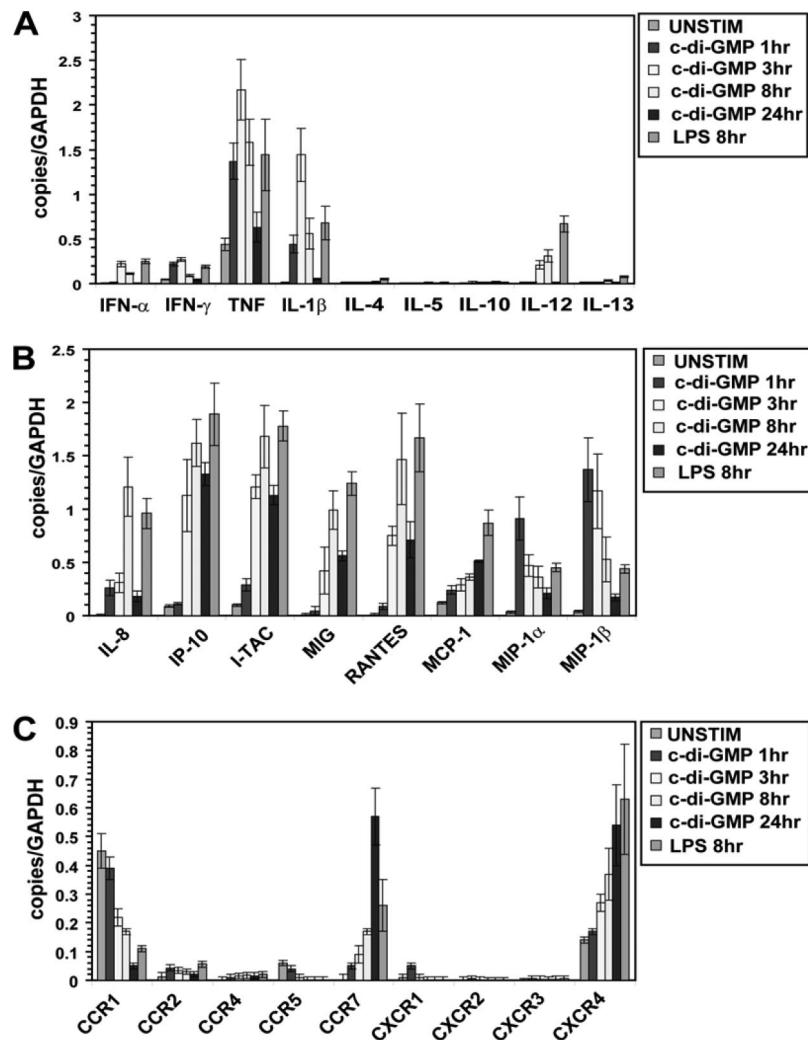


FIGURE 7. Cyokine, chemokine, and chemokine receptor expression during c-di-GMP-induced DC maturation. Expression of cytokines (A), chemokines (B), and chemokine receptors (C) were quantified by QPCR. Total RNA was extracted from 100,000 cells stimulated with 200 μ M c-di-GMP for 1, 3, 8, and 24 h or 100 ng/ml LPS for 8 h and the number of transcripts is normalized to the number of copies of GAPDH. Error bars indicate SD of triplicate measurements.

has in affecting DCs trafficking, we measured expression of chemokine receptors in cells stimulated with c-di-GMP. We found that the chemokine receptors CCR1 and CCR5 were down-regulated, whereas CXCR4 and the lymph node homing receptor CCR7 were up-regulated on DCs following c-di-GMP treatment (Fig. 7C).

To rule out the possibility that the immunostimulatory effects of c-di-GMP were not due to the presence of LPS endotoxin contamination in the synthetic c-di-GMP preparation, we performed a *Limulus* amebocyte lysate assay. We found that the highest dose of c-di-GMP (500 μ M) used in our experiments did not contain LPS (<3 pg/ml LPS).

Effect on human pDCs

Since pDCs represent a separate DC lineage compared to those of myeloid origin, we did test whether c-di-GMP might also activate these cells for cytokine secretion, namely, IFN- α secretion. In two independent experiments done in duplicate, c-di-GMP was negative for inducing this type I IFN. In contrast, both pDC preparations produced IFN- α in response to stimulation with the type A CpG oligodeoxynucleotide ODN-2216 (data not shown).

c-di-GMP enhances T cell stimulatory activity

Another feature of DC maturation is an enhanced ability to stimulate T cells due to increased MHC and costimulatory receptor expression. Therefore, to determine whether c-di-GMP-treated

DCs have an enhanced ability to stimulate T cells, a T cell proliferation assay was performed. Immature DCs, LPS-treated DCs, and c-di-GMP-treated DCs were cocultured with T cells for 6 days, and allospecific T cell proliferation was measured by radiolabeled thymidine incorporation. Fig. 8 shows that T cell proliferation was

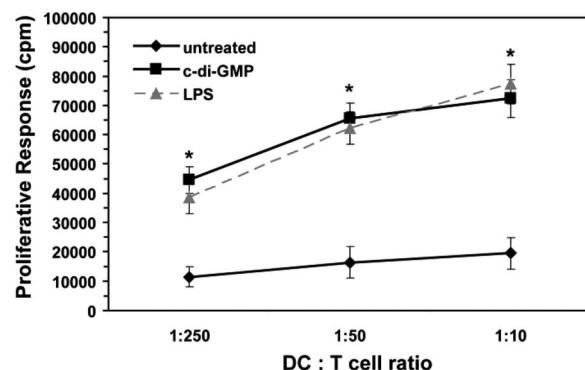


FIGURE 8. c-di-GMP-matured DCs have enhanced T cell stimulatory activity in vitro. Immature DCs were stimulated with 200 μ M for 24 h. Purified T cells were then added to DCs at the ratio indicated and were allowed to incubate for an additional 5 days. On day 6, 1 μ Ci/well [3 H]thymidine was added and allowed to incubate for an additional 18 h. T cell proliferation was measured by [3 H]thymidine uptake (cpm). *, $p < 0.01$, as determined by Student's t test and error bars indicate SD of triplicate measurements.

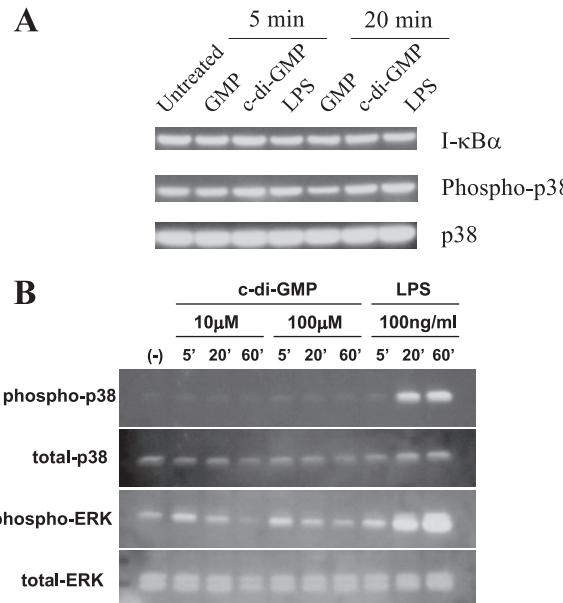


FIGURE 9. c-di-GMP induces signaling in DCs and macrophages. *A*, Activation of p38 MAPK in human DCs. Immature DCs starved overnight were incubated in the absence or presence of c-di-GMP or GMP at specified concentrations (micromolar) for 5 or 30 min. Similar results were found in three independent experiments. *B*, Activation of ERK in human macrophages. Macrophages were exposed to c-di-GMP for 5 min. Following exposure to c-di-GMP, DCs and macrophages were harvested to make cell lysates. Identical amount of cell lysates was separated on a 4–12% gradient PAGE gel, transferred onto a piece of polyvinylidene difluoride membrane, and analyzed by Western blot with the use of Abs.

markedly up-regulated (4- to 7-fold) in cocultures receiving DCs pretreated with c-di-GMP, and this was identical to that seen in cocultures receiving LPS stimulated DCs.

c-di-GMP activation of p38 MAPK in human DCs

To determine whether c-di-GMP could activate intracellular signaling, we investigated the effect of c-di-GMP on the activation of p38 MAPK. As shown in Fig. 9A, c-di-GMP did not activate NF- κ B (since it did not activate I κ B α degradation), but activated p38 MAPK (as indicated by the increase in the phospho-p38 at both 5- and 20-min treatment). LPS and GMP were used as positive and negative controls, respectively. In addition, c-di-GMP did not activate either ERK (data not shown). Since TNF expression is regulated by NF- κ B and we only examined up to 30 min after the addition of c-di-GMP, we cannot rule out activation of NF- κ B at

some later time point. These results were reproduced in four independent experiments using different donor-derived DCs.

Phosphorylated ERK signaling in human macrophages

Based on three independent experiments, although there was no phosphorylation of p38 MAPK detected under the conditions tested, we found that c-di-GMP (10 and 100 μ M) activates ERK phosphorylation in human M-CSF-induced monocyte-derived macrophages (Fig. 9B). The magnitude of c-di-GMP activation of ERK phosphorylation was weaker than that by LPS under the conditions tested. ERK phosphorylation peaked at 5 min and was absent at 20 min, which was earlier than that compared with LPS-induced ERK phosphorylation.

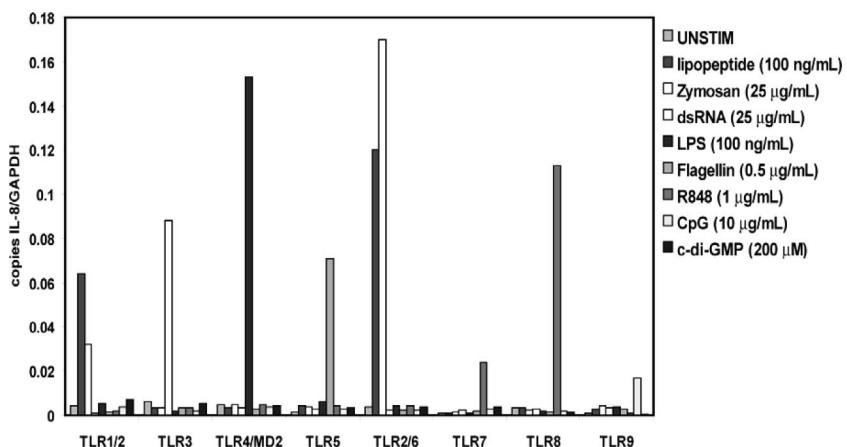
c-di-GMP-induced cell activation is TLR and Nod independent

It has been previously shown that lipopeptides, LPS, dsRNAs, flagellin, ssRNAs, and CpG DNA induce cellular activation via TLRs and that these pathogen-associated factors can induce DC maturation. Because various nucleotide structures have been shown to stimulate DCs through TLRs, we hypothesized that c-di-GMP may also induce cellular activation through a TLR-dependent mechanism. To test this hypothesis, HEK cells, which are normally unresponsive to TLR ligand stimulation, were stably transfected with TLR1/2, TLR3, TLR4/MD2, TLR5, TLR2/6, TLR7, TLR8, and TLR9. As expected, these cell lines responded to TLR ligands according to their TLR expression profile (i.e., TLR3:dsRNA, TLR4/MD2:LPS); however, none of the TLR-expressing cell lines were activated by c-di-GMP (Fig. 10). In addition to our earlier results showing no LPS contamination by the *Limulus* amebocyte lysate assay, by failing to confer TLR responsiveness to c-di-GMP (i.e., c-di-GMP failed to stimulate the LPS-sensitive MD2/TLR4-HEK cell line), the data confirm that the immune-enhancing activity of c-di-GMP is not due to any LPS contamination. Furthermore, we found that neither Nod1 or Nod2 responded to c-di-GMP (data not shown). These results suggest that c-di-GMP immunostimulation does not involve any known TLR or Nod.

c-di-GMP is stable in human serum

HPLC analysis of the stability of c-di-GMP in human serum after 24 h at 37°C showed only one eminent peak due to a nucleotide at the same retention time, which is identical to the retention time of monomeric c-di-GMP (data not shown). These results indicate that 100 μ M c-di-GMP underwent no change in size and two-dimensional structure. Similar results were obtained with 500 μ M c-di-GMP in which the ratio of the peaks due to c-di-GMP and the

FIGURE 10. TLRs fail to respond to c-di-GMP. HEK cells stably transfected with TLR proteins were stimulated for 3 h with 200 μ M c-di-GMP. Cells were harvested for RNA and IL-8 transcripts were quantified by QPCR.



internal standard 2-methylbenzimidazole were ~100%, further indicating that no decomposition of c-di-GMP had taken place in human serum (data not shown). No peaks due to linear GpGp or pGpG and 5'-GMP were detected. The results of the HPLC analysis of human serum only (data not shown) show that three peaks were observed at retention times of ~5, 20, and 50 min. However, these peaks did not overlap with those due to c-di-GMP and 2-benzimidazole used for the internal standard. The internal standard 2-methylbenzimidazole was used because our data show that this compound is fairly soluble in water, methanol, and acetonitrile (solvents used for the preparation of the test solution and HPLC analysis), does not react with c-di-GMP, and is stable in human serum. Overall, these results suggest that c-di-GMP is stable in serum in addition to tissues (e.g., mammary gland).

Discussion

The cyclic dinucleotide c-di-GMP is now recognized as a widespread intracellular signaling molecule in prokaryotes (2, 3, 5–18). We have explored the extracellular biological effects of c-di-GMP and its potential use as a therapeutic agent. We have recently shown that extracellular c-di-GMP specifically inhibits *S. aureus* cell-cell interactions, biofilm formation, and adherence to HeLa epithelial cells in vitro (19). Furthermore, we have shown that c-di-GMP treatment significantly attenuates *S. aureus* infection in a mouse mastitis model (21).

In the current study, we demonstrate that pretreatment of mice with c-di-GMP has a significant protective and prophylactic effect against *S. aureus* infection when c-di-GMP was administered 12 and 6 h before bacterial inoculation in the mouse mammary glands. *S. aureus* infections such as bovine mastitis are difficult to treat and relapsing infections frequently occur (34). The ability of *S. aureus* to enter and survive in phagocytic and nonphagocytic cells has been recognized and often correlates with the high frequency of relapse after antimicrobial therapy (35–37). Also, to date it has been difficult to develop a highly protective vaccine for *S. aureus* (38, 39) and no vaccine that generates both humoral and cell-mediated responses that would be adequate for intracellular pathogens (40–42) is available although DNA-based vaccines are promising (43). Not only does c-di-GMP have a protective effect against *S. aureus* infection but when given along with an Ag as part of a vaccine strategy, c-di-GMP promotes an Ag-specific immune response (adjuvant effect). This immunostimulatory effect is perhaps largely due to the capability of c-di-GMP to induce phenotypic and functional maturation/activation of myeloid DCs, but not pDCs.

Although our results show that DCs release MCP-1 in response to c-di-GMP, we do not believe that DC-derived MCP-1 is recruiting monocytes since MCP-1 release from activated macrophages is a more natural occurrence. As our data suggested that i.p. injection of c-di-GMP can activate monocyte and granulocyte recruitment, we propose that c-di-GMP might activate resident peritoneal macrophages to produce or release monocyte chemoattractants, such as MCP-1.

Consistent with an ability to act as an immunostimulator and immune enhancer, our results show that c-di-GMP stimulates both mouse and human DC maturation and differentiation, Ag-presenting capacity, and enhanced T cell stimulatory activity, leading to an overall Th1 response. The immunostimulatory effect of c-di-GMP is more specific and not as overwhelming as that seen with LPS. Supporting an immune enhancer effect, it is known that IL-8 production results in enhanced migration of DCs and macrophages. The increase in MIG/CXCL9 (a chemoattractant for activated T cells) suggests possible antitumor activity. The ability of c-di-GMP to activate mouse and human DCs is consistent with our findings that pretreatment with c-di-GMP has a significant pro-

tective effect in the mouse model of mastitis and it has significant adjuvant properties when administered with an Ag.

Activation of the MAPK pathways, including p38, ERK, and JNK, are important for the differentiation/maturation of DCs and the NF- κ B transcription factor is linked to the production of proinflammatory cytokines by DCs and has substantial impact on innate and adaptive immunity (44–48). The activation of p38 MAPK has been shown to play a critical role in the activation of DCs in response to a variety of stimuli (49–53). In this study, we tested the hypothesis that the action of c-di-GMP on the host immune response involves modulation of cell signaling pathways. Since c-di-GMP did not activate ERK and JNK in DCs under the conditions tested, the exact mechanism by which c-di-GMP activates p38 MAPK awaits further investigation. However, the ability of c-di-GMP to activate p38 MAPK in DCs is consistent with its ability to induce the maturation of DCs, as evidenced by the up-regulation of DC surface costimulatory molecules, cytokines, chemokines, and chemokine receptors, and its capacity to stimulate allogeneic MLR.

Our results showing that mouse i.p. injection of cyclic dinucleotide activates recruitment of monocytes and granulocyte recruitment suggested that c-di-GMP-activated resident macrophages may produce and release monocyte chemoattractants, such as MCP-1 (MCP-1/CCL2) (54). MCP-1 plays a major role in the recruitment of monocytes into inflammatory sites and its production is dependent on activation of p38 MAPK (55, 56). Based on the ability of c-di-GMP to activate monocytes and granulocytes in vivo, we examined whether c-di-GMP could activate human monocyte-derived macrophages in vitro by evaluating the phosphorylation of two MAPKs, ERK and p38 MAPK, in response to c-di-GMP. However, although we detected a transient phosphorylation of ERK in macrophages, we detected no obvious change in p38 MAPK phosphorylation in macrophages under the conditions tested. One possible explanation for this difference is that macrophages (p38 MAPK is not induced) and DCs (p38 MAPK is induced) respond differently to c-di-GMP. Because we found no effect on p38 MAPK in macrophages, our results suggest that the in vivo recruitment of monocytes, granulocytes, and macrophages by c-di-GMP does not result from production of MCP-1.

One primary host mechanism for detection of pathogens or microbial products (“danger signals”) is mediated by plasma membrane-bound pathogen recognition receptors called TLRs. TLRs interact with various microbe-associated molecules, and subsequent activation induces up-regulation of costimulatory molecule expression, production of antimicrobial effector molecules, and secretion of proinflammatory cytokines and chemokines. However, it is also possible that instead of innate immune signaling at the cell surface, signaling might occur in the cytosol in a TLR-independent manner. An emerging family of pathogen recognition receptors, called Nods, also appear to be involved in the recognition of bacterial products and can trigger an innate immune response. Nods are cytosolic proteins involved in innate immune defense through pathways that are likely to be independent of TLR signaling. Although c-di-GMP is an immunostimulatory molecule derived from bacteria, our TLR and Nod tests suggest that immune activation does not appear to involve TLRs 1–9 or Nods 1 and 2. In addition, the pDC data also indicate that TLR9 is not likely involved. Therefore, although the exact receptor-mediated mechanism of c-di-GMP immune activation is not yet known, it does not act through any currently known TLR or Nod.

If cyclic dinucleotides (like c-di-GMP) are to be used clinically, it is important to know their stability in vivo since serum might contain mammalian phosphodiesterases that could potentially cleave and degrade the molecule. Our results clearly demonstrate

that c-di-GMP is stable in pooled human serum and is an advantageous property for potential clinical use.

The data presented in the current study show that bacterial c-di-GMP is an immunostimulatory molecule and we have previously demonstrated that c-di-GMP can inhibit bacterial infection (19, 21) and has potential in cancer treatment (20). Due to its microbial origin and its effect on the host response, c-di-GMP can be considered as a danger signal or novel pathogen-associated molecular pattern. Our findings with c-di-GMP have some features in common with other immunostimulatory molecules. Bacterial DNA containing unmethylated CpG motifs is also known to stimulate production of polyclonal Ig and Th1-associated immunomodulatory cytokines including IFN- γ , IL-12, and TNF- α , which provide some protection against intracellular pathogens (39–41). CpG-containing oligodeoxynucleotides, which act as TLR9 agonists, are recognized as adjuvants modulating mucosal immune responses (57). Like CpG, c-di-GMP itself can stimulate an immune response; however, the immunostimulatory ability of c-di-GMP is TLR independent. Our results show that c-di-GMP also enhances a Th1 response: 1) pretreatment of mice with c-di-GMP inhibits bacterial infection, 2) c-di-GMP preferentially promotes the Ag-specific IgG2a over IgG1, 3) c-di-GMP up-regulates IL-12, not IL-10, and 4) c-di-GMP preferentially stimulates p38 MAPK in DCs.

A potential mechanism of action is that c-di-GMP may interact with a putative receptor, trigger an intracellular signal transduction cascade resulting in the up- and down-regulation of genes leading to the mobilization and activation of monocytes and granulocytes and DCs, and hence the inhibition of infection and enhancement of Ag-specific immune responses. Our data do indicate the triggering of intracellular signal transduction cascades. However, the exact receptor (membrane or intracellular) c-di-GMP utilizes is currently under investigation; our data rule out the involvement of all known TLRs and Nods.

In human and animal vaccine development, a major drawback to the development of novel vaccines has been the lack of safe, yet effective adjuvants. For example, biphasic lipid vesicles have been proposed for the delivery of oligo/polynucleotides (58). The demonstration that the c-di-GMP acted as an adjuvant for vaccination with *S. aureus* ClfA Ag when coinjected i.m. indicates good bioavailability without the requirement of specific delivery systems or formulations. In addition to its use alone in preventing infection, it will be interesting to see whether c-di-GMP coadministered with other Ags or as part of a vaccine preparation improves protection against subsequent challenge.

The inhibition against experimental *S. aureus* infection in mice clearly establishes a potential clinical use for the protective effect of c-di-GMP against infection (prevention). Another potential use is for increasing the immunity status of individuals or a population either at known risk of developing disease to reduce infection (“metaphylaxis”) or during periods of known disease susceptibility or immune suppression (“immune restoration”). Since c-di-GMP targets the host immune response, these molecules would also be protected from most of the bacterial resistance mechanisms found in microorganisms that affect specific classes of antibiotics. We propose that cyclic dinucleotides, such as c-di-GMP, represent a new class of immunotherapeutic molecules.

In conclusion, this study constitutes a novel investigation into the biological effects of c-di-GMP on the immune response and its potential clinical use. Our findings from various in vivo models (infection, adjuvant, and monocyte and granulocyte recruitment) using different routes of administration (intramammary, i.m., and i.p.) demonstrate that c-di-GMP is an immunostimulatory molecule triggering innate and adaptive immune responses. We also

show, using in vitro models, that c-di-GMP activates human monocyte-derived DCs and acts like an adjuvant. We propose that cyclic dinucleotides like c-di-GMP have broad activity and can be used clinically in humans and animals as an immune enhancer, immunotherapeutic and immunoprophylactic agent, or as a vaccine adjuvant to inhibit infection and disease.

Acknowledgments

We are very grateful to Marcela Pasetti and Matthew Augustine for reviewing this manuscript and providing helpful comments. We also thank Caroline Bergeron for technical assistance with mice in the prophylaxis and vaccine adjuvant studies.

Disclosures

D. K. R. Karaolis has three related patents: method for attenuating virulence of microbial pathogens and for inhibiting microbial biofilm formation (PCT/US04/23498), method for stimulating the immune, inflammatory, or neuroprotective response (U.S. 11/079, 886; PCT/US05/08447), and method for inhibiting cancer cell proliferation or increasing cancer cell apoptosis (U.S. 11/079,779; PCT/US05/08448).

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